

# Calmodulin activates prokaryotic adenylate cyclase

(*Bordetella pertussis*/trifluoperazine/troponin C/oxidized calmodulin)

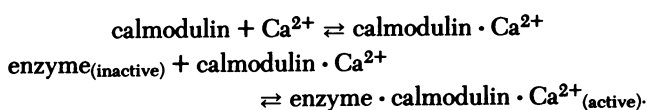
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**ABSTRACT** The adenylate cyclase of *Bordetella pertussis* is stimulated 100- to 1000-fold in a dose-dependent manner by calf brain calmodulin. The system has the following properties. (i) The activation is prevented by ethylene glycol bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid and restored by  $\text{Ca}^{2+}$ . (ii) Oxidation of the methionine residues of calmodulin abolishes the ability to activate the cyclase. (iii) Trifluoperazine inhibits calmodulin-activated cyclase. (iv) A troponin C preparation stimulates the *B. pertussis* cyclase with <0.01 the potency of calmodulin. Although calmodulin has not been demonstrated in prokaryotes, this is an example of a (eukaryotic) calmodulin effect in a prokaryote.

Adenylate cyclase of *Bordetella pertussis* has been shown to require a protein activator for optimal activity (1, 2). Its remarkable heat stability suggested a resemblance to an activator of mammalian brain adenylate cyclases, calmodulin (or calcium-dependent regulator). This small protein, 16,700 daltons, interacts with a large number of enzymes and proteins in a  $\text{Ca}^{2+}$ -dependent manner by a two-step mechanism that may be depicted as follows (3-6):



The relatively few adenylate cyclases so far shown to be regulated by calmodulin are from neural tissues (7-14), adrenal medulla (15), and pancreatic islets (16). It seemed reasonable, therefore, to investigate the effect of this regulator protein on *B. pertussis* adenylate cyclase. The present study shows activation of this enzyme up to 100- to 1000-fold and is an example of such an effect of calmodulin in a prokaryote.

## METHODS

**Growth of the Organism.** *B. pertussis* (strain 114) organisms were grown from a 5% inoculum in 250 ml of modified Stainer-Scholte medium (in 500-ml flasks) on an oscillating shaker at 35.5°C with room air as the gas phase as described (17); incubation was for 20-24 hr. The flasks were then stored at 4°C and the suspension was used for several weeks unless otherwise indicated. Under these conditions, basal adenylate cyclase activity increased with time but the maximal stimulation attainable did not change.

Supernatant culture medium which contains 0.1-0.2 of the total cyclase activity (18) was obtained by centrifugation of the suspension at  $5000 \times g$  for 10 min. Washed cells were prepared from the cell pellet by resuspending in 0.154 M NaCl that had been passed through a Chelex-100 column to remove divalent cations. This was repeated four times.

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**Adenylate Cyclase Assay.** Because a substantial fraction of the total adenylate cyclase of this organism is found in the periplasmic space and can be assayed with exogenous ATP (18), intact organisms were used in most assays. The reaction was carried out at 30°C by the method of Salomon *et al.* (19) in 60  $\mu\text{l}$  containing 60 mM *N*-tris(hydroxymethyl)methylglycine-HCl (pH 7.8), 10 mM  $\text{MgCl}_2$ , 1 mM [ $\alpha$ - $^{32}\text{P}$ ]ATP ( $1-1.5 \times 10^6$  cpm), and 10  $\mu\text{l}$  of the bacterial suspension. GTP has no effect on this enzyme and was not included. The order of addition of the components of the assay mixture proved to be of importance. In some experiments, bacteria and activator were preincubated for 10 min and then the reaction was started by addition of the prewarmed substrate and was allowed to run for 10 min as described (1, 2). In most assays, the bacteria were added last to start the reaction as described in the legends.

**Preparation of Calmodulin.** Calmodulin was prepared from calf brains as described by Klee (20) with the following modifications: (i) the starting material was the first warm high-speed supernatant from microtubule preparations (see ref. 21 for details on procedures used for obtaining this supernatant), and (ii) the gel filtration step used a composite column (5  $\times$  68 cm) composed, from top to bottom, of 31 cm of Sephacryl S-200 (superfine), 6 cm of Sephadex G-25 (superfine), and 31 cm of Sephadex G-75 (medium). In addition, we tested a preparation of calmodulin generously supplied by C. Klee (National Cancer Institute). Oxidation of the methionine residues of calmodulin with chloramine-T was carried out according to the procedure of Walsh and Stevens (22).

**Materials.** Troponin C was the generous gift of Robert Adelstein. Trifluoperazine was a gift of Smith, Kline & French. Other chemicals were obtained as follows: *N*-tris(hydroxymethyl)methylglycine, from Calbiochem; [ $\alpha$ - $^{32}\text{P}$ ]ATP,  $\approx 25$  Ci/mmol (1 Ci =  $3.7 \times 10^{10}$  becquerels), from ICN; cyclic [ $^3\text{H}$ ]AMP, from New England Nuclear; ATP (type II), from Sigma.

## RESULTS AND DISCUSSION

Calf brain calmodulin stimulated the adenylate cyclase activity of intact *B. pertussis* organisms (Table 1). The stimulation was dose-dependent and did not require the addition of exogenous  $\text{Ca}^{2+}$  to the system because substantial amounts of  $\text{Ca}^{2+}$  were derived from the Stainer-Scholte medium ( $\approx 30 \mu\text{M}$   $\text{Ca}^{2+}$  in assay). Repeated washing of the organisms with 0.154 M NaCl that had been passed through a Chelex-100 column to remove divalent cations did not decrease the activity and generally increased the specific activity. On the other hand, 1 mM ethylene glycol bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) essentially abolished the stimulation attainable with calmodulin: inhibition at 60 nM calmodulin was 96% for the cell suspension, 98% for the washed cells, and 72% for the

Abbreviation: EGTA, ethylene glycol bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

Table 1. Effect of EGTA and calcium on calmodulin-stimulated adenylate cyclase preparations of *B. pertussis*

Enzyme source	Added calmodulin, nM	Adenylate cyclase, nmol cyclic AMP/min/mg			
		Control	Plus EGTA (600 $\mu$ M)	Plus $\text{Ca}^{2+}$ (900 $\mu$ M)	Plus $\text{Ca}^{2+}$ and EGTA
Whole cell suspension	0	0.49	0.41	0.45	0.51
	60	45.0	1.92	42.1	41.3
	600	98.5	—	—	—
Washed organisms	0	0.36	0.25	0.27	0.30
	60	48.2	1.02	47.4	46.1
	600	111.3	—	—	—
Supernatant enzyme	0	0.36	0.30	0.22	0.29
	60	4.69	1.28	4.59	3.67
	600	12.6	—	—	—

Standard incubation conditions; reaction was started by addition of enzyme (6.1  $\mu$ g of protein for the crude suspension, 4.3  $\mu$ g for the washed cells, and 1.9  $\mu$ g for the culture supernatant).

supernatant enzyme. The inhibiting effects of EGTA could be prevented by excess  $\text{Ca}^{2+}$ . The concentrations of  $\text{Ca}^{2+}$  used were themselves slightly inhibitory for all three enzyme preparations for both basal and calmodulin-stimulated activities. At 2 mM  $\text{CaCl}_2$  this inhibition increased (data not shown). This is typical of many mammalian adenylate cyclases whether they are calmodulin-sensitive or not. Whether or not basal activity, measured without added calmodulin, was intrinsic or a result of contamination by an activator could not be readily determined by using intact organisms.

The crude supernatant solution from *B. pertussis* organisms also contains adenylate cyclase activity and, in some preparations, this amounts to 15–20% of the total adenylate cyclase of the system (18). The supernatant enzyme was also stimulated by calmodulin and also showed little effect of added  $\text{Ca}^{2+}$  (Table 1). Stimulation was always less than with intact organisms and varied from 3- to  $\approx$ 35-fold (35-fold in Table 1) with 600 nM calmodulin whereas the cells were stimulated 200-fold and washed cells 310-fold at this concentration. In fact, in some fresh preparations of *B. pertussis*, stimulation by 600 nM calmodulin exceeded 1000-fold (data not shown). This effect was entirely on the  $V_{\max}$  and no change in the  $K_m$  for ATP could be detected (data not shown). This has already been shown for the brain cyclase (10). Although the culture supernatant showed substantial, albeit decreased, activation by calmodulin (Table 1), the purified enzyme did not respond to activation by calmodulin plus  $\text{Ca}^{2+}$ , and we attribute this to the loss of another factor needed by the system. This will be the subject of another report.

Inhibition with EGTA was studied at constant total calcium concentrations. Increasing EGTA yielded titration curves that showed midpoint displacements proportional to the added  $\text{Ca}^{2+}$  (Fig. 1). Without  $\text{Ca}^{2+}$  the curve reflects contamination from the assay system. It should be noted that the slopes were steeper at higher  $\text{Ca}^{2+}$  concentration.

The affinity of calmodulin for the adenylate cyclase system of *B. pertussis* is shown in Fig. 2 for two separate preparations of the activator protein. Half-maximal stimulation occurred at 45 nM calmodulin for both preparations, and this value was not altered by the addition of 100  $\mu$ M  $\text{Ca}^{2+}$  to the incubation mixture. Furthermore, this value was not changed significantly

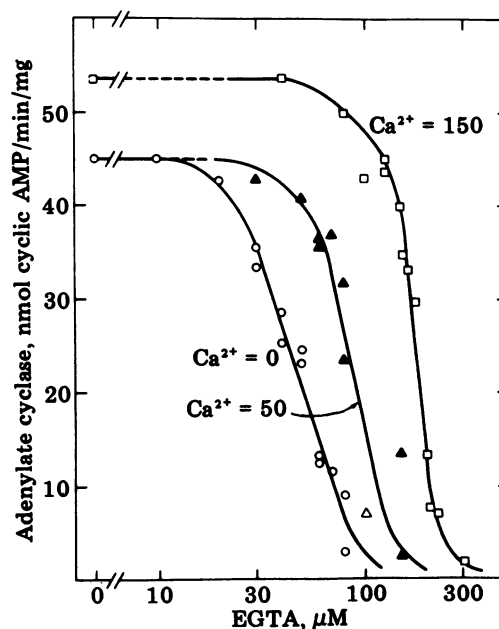


FIG. 1. Inhibition of calmodulin-stimulated adenylate cyclase by EGTA; standard incubation conditions with 60 nM calmodulin. In the absence of calmodulin, basal values were 0.25–0.3 nmol cyclic AMP/min per mg and were not affected by EGTA (data not shown).  $\text{Ca}^{2+}$  concentrations refer to added  $\text{CaCl}_2$  (as  $\mu$ M). The reaction was started by addition of washed organisms. All points are means of triplicate determinations. A second point at a single EGTA concentration represents replicate experiments made the next day.

when 1/10th the amount of *B. pertussis* organisms (0.6  $\mu$ g of protein) was used in the assay (data not shown). The system was very sensitive, and a doubling of basal activity occurred at  $\approx$ 1 nM calmodulin. This implies that  $<1$  pmol of calmodulin can be determined with reasonable accuracy. Hence, this organism may prove to be useful in an assay for calmodulin.

Although stimulation occurred at low concentrations of the calmodulin preparations, it seemed important to rule out the possibility of a contaminant despite apparent purity in Na-DodSO<sub>4</sub>/polyacrylamide slab gel electrophoresis. One way to accomplish this was by chemical modification of the protein. Although most such modifications of calmodulin have little

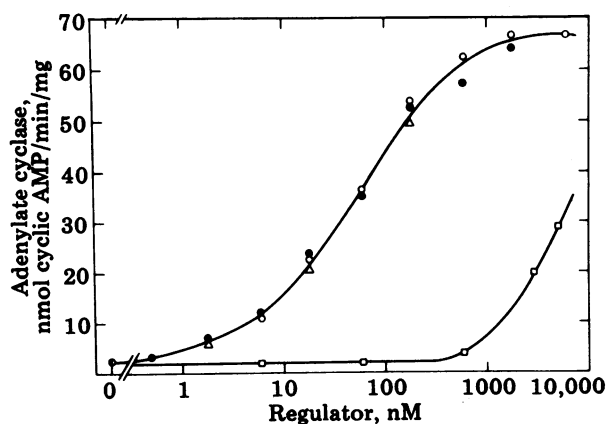


FIG. 2. Concentration-dependence of adenylate cyclase activation by calmodulin and oxidized calmodulin; standard incubation conditions. The reaction was started by addition of microorganisms (6.5  $\mu$ g of protein). The concentration of  $\text{Ca}^{2+}$  was 30  $\mu$ M except for curve  $\Delta$  it was 130  $\mu$ M. Preparation K was generously provided by C. Klee. O, Preparation SB.1;  $\bullet$ , preparation K;  $\Delta$ , preparation K +  $\text{Ca}^{2+}$ ;  $\square$ , oxidized calmodulin.

effect on the activation of phosphodiesterase, oxidation of methionine residues is known to abolish activator activity (22). Accordingly, we oxidized calmodulin with chloramine-T, dialyzed the preparation, and tested it for activation of adenylate cyclase activity in intact *B. pertussis* organisms. As shown in Fig. 2, greater than 99% of the potency of the preparation was destroyed by oxidation. Addition of  $\text{Ca}^{2+}$  (to 100  $\mu\text{M}$ ) did not enhance the activity.

One of the interesting properties of calmodulin is its ability to bind phenothiazines, with consequent blockade of the stimulatory effect of calmodulin—e.g., on phosphodiesterase (23) and adenylate cyclase (24, 25). Trifluoperazine was found to block calmodulin-stimulated adenylate cyclase in intact *B. pertussis* organisms with an apparent  $K_i$  of  $\approx 50$   $\mu\text{M}$  (Fig. 3). Additional  $\text{Ca}^{2+}$  (100  $\mu\text{M}$ ) did not enhance the potency of this inhibitory effect. The concentration required is considerably higher than the binding constant of trifluoperazine for pure calmodulin (23), is slightly higher than that reported for the inhibition of phosphodiesterase (10  $\mu\text{M}$ ) (23), and is lower than that for adenylate cyclase activities from thyroid membranes (23) or brain particulates (25). Because whole organisms were used, the effective concentrations of trifluoperazine may have been lower. Moreover, a direct effect on the cyclase can not be ruled out (24, 26).

The fact that calmodulin resembles troponin C in a number of ways and can activate certain enzymes in a similar (though less sensitive) way (3–6) led us to try troponin C as activator of *B. pertussis* adenylate cyclase. Clear-cut activation could be elicited but occurred with a potency <1% that of calmodulin (Fig. 4). This effect could also be abolished by trifluoperazine (100  $\mu\text{M}$ ), and the possibility of contamination of this preparation with small amounts (<1%) of calmodulin is difficult to rule out.

During the course of these studies, certain findings suggested that the interaction of calmodulin with the *Bordetella* adenylate cyclase system differed from that seen with the cyclase from brain (7, 8). The order of addition of EGTA with respect to calmodulin proved to be important. When the chelator was added before or simultaneously with calmodulin, it prevented the stimulation of adenylate activity (Table 1). However, when the activation was allowed to proceed before EGTA was added,

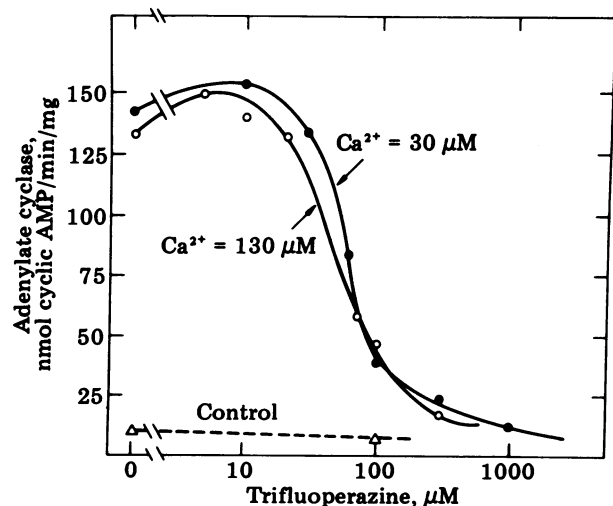


FIG. 3. Effect of trifluoperazine on calmodulin-activated adenylate cyclase of *B. pertussis*. Standard incubation conditions were used with 600 nM calmodulin except in the controls. In some experiments,  $\text{CaCl}_2$  was added to yield a total of  $\text{Ca}^{2+}$  concentration of  $\approx 130$   $\mu\text{M}$ . *B. pertussis* (7.3  $\mu\text{g}$  of protein) was added to start the reaction, which was run at 30°C for 10 min.

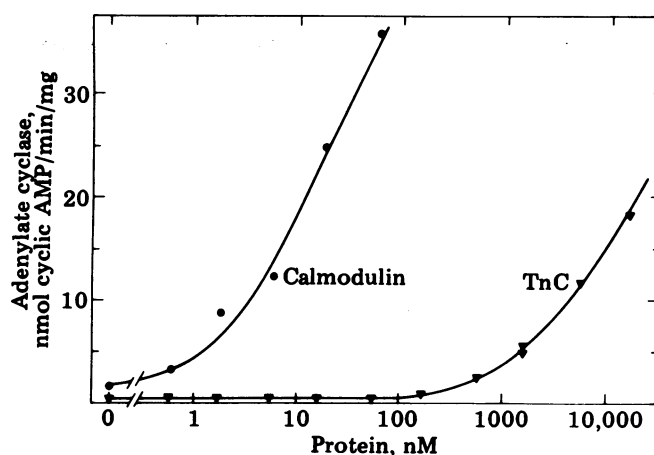


FIG. 4. Comparison of the potencies of calmodulin and troponin C (TnC) in the activation of adenylate cyclase of *B. pertussis*. Standard assay conditions were used except that  $\text{Ca}^{2+}$  concentrations were 33  $\mu\text{M}$  for the calmodulin curve and 100  $\mu\text{M}$  for the troponin C curve. The reaction was started by addition of *B. pertussis* and was run at 30°C for 10 min.

there was relatively little reduction in the accelerated rate of cyclic AMP generation (Fig. 5). Moreover, there was a latency of 2–3 min before the change in rate of cyclic AMP accumulation became manifest. For some experiments, no significant decrement could be demonstrated (data not shown). In the same preparation, 1 mM EGTA present from the start caused a >95% inhibition of stimulation. These results differ from the marked decreases in rate occurring without significant latency seen in brain cyclase (7, 8) and suggest either that the binding between the calmodulin- $\text{Ca}^{2+}$  complex and *B. pertussis* is of high affinity and slowly reversible or that the  $\text{Ca}^{2+}$  in the enzyme-calmodulin complex becomes sequestered such that it is not as readily available for chelation. These effects resemble the EGTA-resistant binding of calmodulin known to occur in phosphorylase kinase (27) wherein the activator constitutes the  $\delta$  subunit of the enzyme.

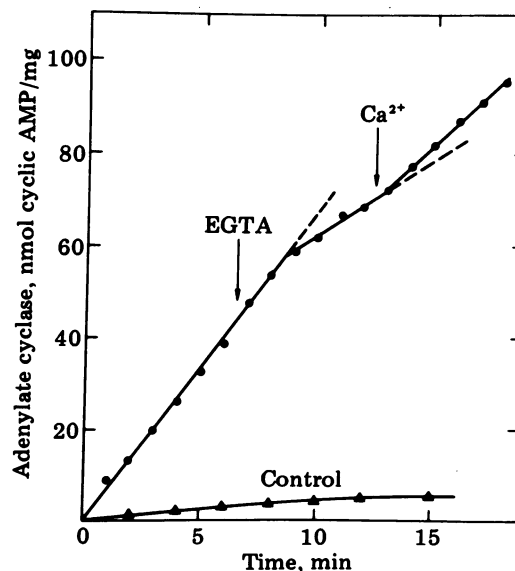


FIG. 5. Reversal of calmodulin activation of cyclase by EGTA. Standard assay conditions were used with starting total  $\text{Ca}^{2+}$  of 30  $\mu\text{M}$ . Calmodulin (60 nM) was preincubated with *B. pertussis* at 30°C for 5 min, and the prewarmed reaction mixture was then added to start the reaction. Duplicate samples were removed every min. At 6.5 min, EGTA was added to a final concentration of 1 mM. At 12.5 min,  $\text{CaCl}_2$  was added to a final total concentration of 1.05 mM.

Whatever the precise nature of the interaction of calmodulin with *B. pertussis* adenylate cyclase may turn out to be, the above data on the highly sensitive stimulation of adenylate cyclase with apparently pure calmodulin, the near total abolition of stimulating activity by oxidation of methionine residues of calmodulin, the  $\text{Ca}^{2+}$  dependence of the activation as shown by EGTA titration, and the inhibition of the activated cyclase by trifluoperazine, all suggest that calmodulin is indeed an activator for adenylate cyclase in *B. pertussis*. The *Bordetella* system thus may be a useful model for the calmodulin interaction with adenylate cyclases.

Although calmodulin activity appears to be widely distributed in eukaryotic cells [from mammals to mushrooms (3–6)], the search for it in bacteria has been without success so far (28). The high activation of adenylate cyclase of *B. pertussis* with exogenous calmodulin shown above suggests that a functional calmodulin-like material probably is absent from this organism as well. The present results, which demonstrate a calmodulin effect in a prokaryote, suggest the possibility that the ability to respond to this regulator may have developed before the ability to synthesize it. Alternatively, the ability to respond to calmodulin may have evolved to facilitate the parasitic state of the organism in the respiratory epithelium.

We thank Dr. Claude B. Klee of the National Cancer Institute for introducing us to the wonders of calmodulin and advising us on its purification and Bruce Meade of the Bureau of Biologics for assistance in growing the organisms.

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